Mechanisms of Cell Death Induced by Tumor Necrosis Factor Antagonists

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INTRODUCTION

TNF Antagonists

- Tumor necrosis factor (TNF) antagonists have been shown to be efficacious in the treatment of several autoimmune diseases, including rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, and psoriasis.
- There are currently 2 classes of biologic drugs that target TNF bioavailability: soluble TNF receptors (etanercept) and anti-TNF monoclonal antibodies (adalimumab and infliximab).
- All 3 currently approved agents bear the Fc portion of complement-activating human IgG1; the Fc region is a native component of the monoclonal antibodies, whereas it is genetically fused to the soluble receptor.

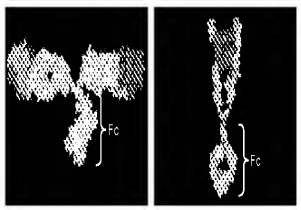
Fc Components of TNF antagonists

- Fc regions bind to Fc receptors (FcγR), which are a family of immunoglobulin-binding molecules expressed on immune cells, including macrophages, granulocytes, natural killer cells, B cells, and platelets.
- Antibody-dependent cellular cytotoxicity (ADCC) is mediated by cross-linking of FcγR.
- Complement-dependent cytotoxicity (CDC) may be enhanced by the cross-linking of Fc, which increases affinity for the complement component C1q.

OBJECTIVE

To assess the ability of infliximab, adalimumab, and etanercept to bind to membrane-bound (mTNF) and the ability of infliximab and etanercept to induce ADCC and CDC.

Figure 1. Models of TNF Antagonists Bound to TNF



Anti-TNF antibody
adalimumab
infliximab

Soluble TNF receptor
etanercept

TNF antagonists are shown in white, and TNF trimers are blue, green, and red. The TNF-binding site of the anti-TNF antibody is shown in cyan.

METHODS

- Construction of mTNF-expressing Chinese Hamster Ovary (CHO) cells
  - A mutant TNF that is expressed in membrane-bound form was constructed by site-directed mutagenesis of the wild-type TNF DNA sequence to remove amino acids 77 through 88.
  - The mutated TNF DNA sequence was cloned into a lentiviral expression vector, which was used to transduce CHO cells.
  - CHO cells expressing mTNF were isolated with a fluorescence-activated cell sorter, using a fluorochrome-conjugated anti-TNF antibody.
  - A high-expressor clone (MT-3) was expanded using selective media and used for all experiments.
- Binding of TNF antagonists to mTNF
  - Fluorescein isothiocyanate-conjugated drug (0.3 µg) was incubated with MT-3 cells alone or in the presence of 300-fold excess soluble TNF for 1 hour at 4°C.
  - The cells were washed with flow buffer (phosphate-buffered saline with 0.2% bovine serum albumin) to remove unbound drugs.
  - Binding of TNF antagonists to cells was analyzed using FACScalibur.
- ADCC assays (performed in triplicate)
  - MT-3 cells were detached from tissue culture flasks with cell dissociation buffer, washed, and labeled with a membrane integrating dye PKH67.
  - MT-3 cells (0.5×10^6 cells) were incubated with varying concentrations of anti-TNF molecules at 4°C for 30 minutes.
  - Purified donor peripheral blood mononuclear cells were mixed with the treated cells at 10:1 or 40:1 ratios for 4 hours.
  - Propidium iodide (binds to the chromatin of dead or dying cells) was added to each well.
  - The degree of cell death was measured by flow cytometry based on the degree of fluorescence from propidium iodide bound to cellular chromatin.
- CDC assays (performed in triplicate)
  - MT-3 cells were detached from tissue culture plates using cell dissociation buffer to form a single-cell suspension.
  - MT-3 cells (0.5×10^6 cells) were incubated in the presence of etanercept or infliximab at the indicated concentrations for 1 hour at 4°C to allow binding to mTNF.
  - Heat-inactivated fetal bovine serum (negative control), complement component C5-depleted human serum (negative control), or human complement-rich serum was added at a final concentration of 10%.
  - Cells and TNF antagonists were incubated in the presence of complement for 3 hours at 37°C.
  - The degree of cell death was determined by analysis of propidium iodide uptake using flow cytometry.

RESULTS

Figure 2. Binding of Etanercept, Adalimumab, and Infliximab to mTNF

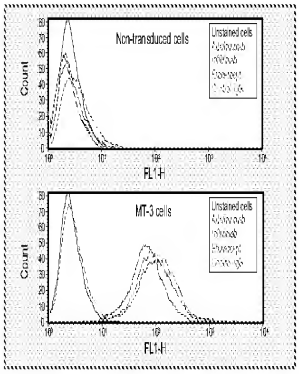


Figure 3. ADCC by Etanercept and Infliximab

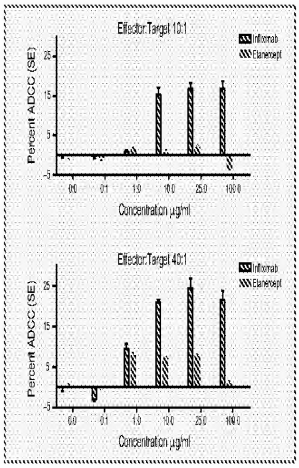
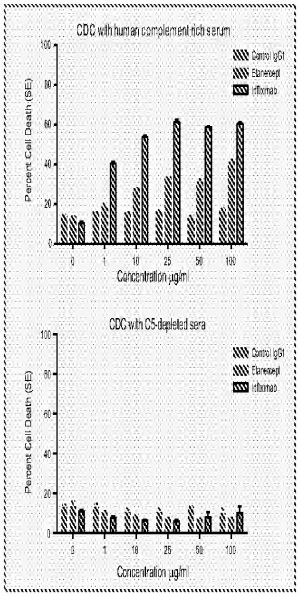


Figure 4. CDC by Etanercept and Infliximab



DISCUSSION

- Soluble receptors and monoclonal antibodies that target TNF decrease levels of bioactive TNF. Both classes of drugs demonstrate efficacy in the treatment of several autoimmune diseases.
- In prior studies, we found that both classes of TNF antagonists bound poorly to FcγR and C1q on cells. In the presence of TNF, anti-TNF monoclonal antibodies, but not the soluble TNF receptor, increased binding to FcγR and C1q.
- Current experiments showed that the anti-TNF antibody infliximab was more effective at inducing ADCC and CDC pathways in mTNF-expressing cells than the soluble TNF receptor etanercept, perhaps via binding to FcγR and C1q.
- These differences in the ability to induce ADCC and CDC pathways may explain the varying spectrum of disease states for which these agents are effective treatments.
- Furthermore, these differences may contribute to the higher rates of fungal and granulomatous infections, such as tuberculosis, observed with infliximab compared with etanercept.[1,2]

CONCLUSIONS

- Both of the TNF antagonists tested, the soluble TNF receptor etanercept as well as the anti-TNF monoclonal antibody infliximab, bound to mTNF on CHO cells.
- Infliximab induced ADCC at lower effector:target ratios than etanercept.
- Infliximab was more effective than etanercept at inducing CDC in mTNF-expressing cells.

REFERENCES

1. Wallis RS, Broder MS, Wong JY, Hanson ME, Beenhouwer DO. Granulomatous infectious diseases associated with tumor necrosis factor antagonists. *Clin Infect Dis.* 2004;38:1261-1265.
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Thanks to Randal Ketchem for molecular imaging studies.

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EXHIBIT M

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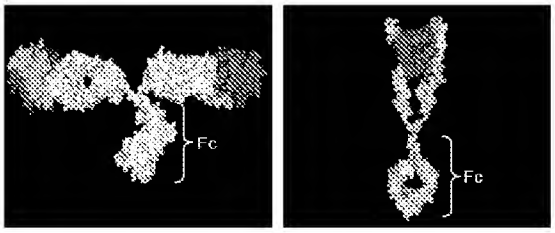

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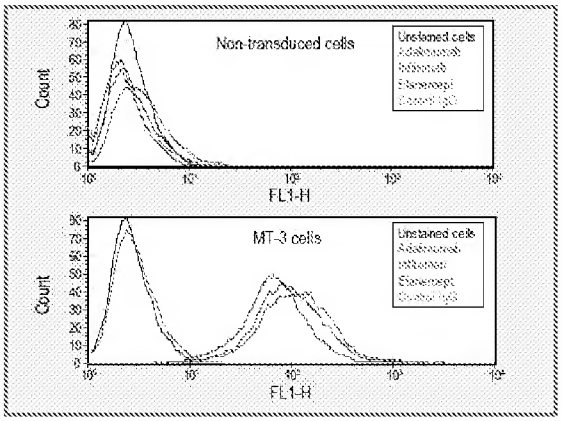

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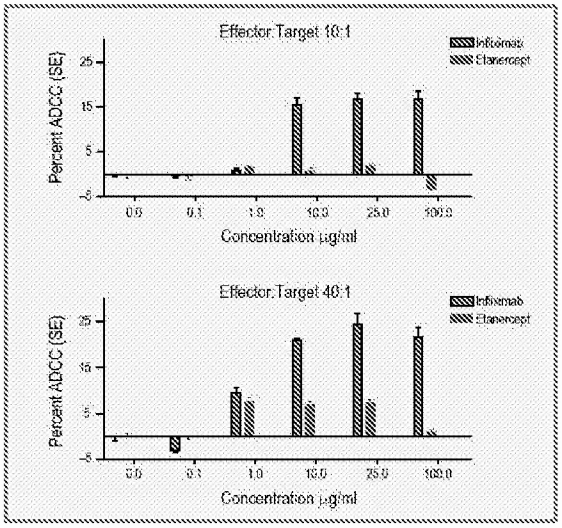

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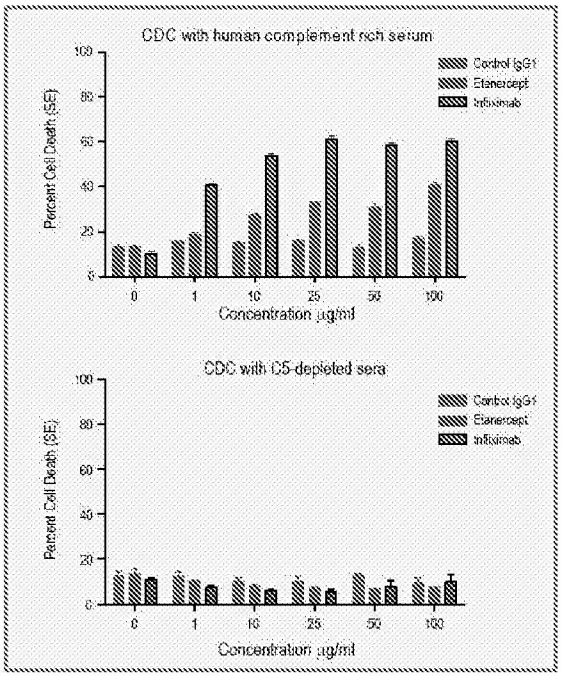

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